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Gene transfer *in vivo*: Sustained expression and regulation of genes introduced into the liver by receptor-targeted uptake

(asialoglycoprotein receptor/gene therapy/human factor IX gene/phospho*enol*pyruvate carboxykinase gene promoter)

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ABSTRACT **Receptor-mediated gene transfer has been used to introduce genes into tissues of animals *in vivo*. The genes introduced by this approach have been transiently expressed at low levels in animal tissues. High levels of expression, for longer periods, have been attained by the induction of cell division (i.e., partial hepatectomy) or disruption of lysosomal degradation of the DNA. We have studied the correlation of specific structural features on the DNA/ligand complexes with their ability to efficiently introduce DNA into the livers of intact animals. A chimeric gene containing the phospho*enol*pyruvate carboxykinase gene promoter (nucleotides −460 to +73) linked to the structural gene for human factor IX (PEPCK–hFIX gene) was condensed with galactosylated poly(L-lysine) by titration with NaCl, resulting in complexes of defined size (10–12 nm in diameter) and shape. The PEPCK–hFIX gene complex was injected into the caudal vena cava of adult rats and the conjugated DNA was specifically targeted to the livers of the animals; no detectable DNA was noted in other tissues. The plasmid containing the PEPCK–hFIX gene was found as an episome in the livers of the rats 32 days after injection of the DNA complex. Human factor IX DNA, mRNA, and functional protein were detected up to 140 days after administration of the DNA complex (the duration of the experiment). Transcription from the PEPCK promoter could be induced over the entire course of the experiment by feeding the rats a high-protein, carbohydrate-free diet. We conclude that the structure of the DNA/ligand complexes is of key importance for the successful introduction of genes into the tissues of animals by receptor-mediated endocytosis.**

Receptor-mediated gene transfer has been used to deliver genes to the liver and other organs (1–5) and affords the advantages of targeting a specific tissue in an adult animal without the use of infectious vectors. However, the approaches used to introduce DNA into animals have the major disadvantage of transient, low-level expression of the transgene (3, 6). Thus, there is a need for a procedure that will result in prolonged expression of the transgene in the target tissue without drastic manipulations, such as partial hepatectomy. Perhaps the major factor which prevents the wider use of receptor-mediated gene delivery in gene therapy is its high degree of variability (5). Because of this variability it is necessary to better characterize the individual components of the DNA/ligand complex and to understand the nature of their assembly into a dependable vehicle for gene delivery. A key step in the assembly of a productive DNA complex capable of efficient gene transfer *in vivo* is condensation of the DNA to a size suitable for uptake via an endocytic pathway. Adjusting the charge neutralization of the DNA molecules by the use of polycations such as poly(L-lysine) results in condensation and eventually precipitation of the DNA in the form of compact structures (7, 8). These perturbations result in a change in the conformation of the DNA molecule allowing the flexible polymer to bend and become compact. The behavior of DNA/polycation complexes in solution is dependent on the method for complexing DNA with the carrier molecule—e.g., poly(L-lysine) (7–11).

There are relatively few structure–function studies involving the internalization of specific DNA structures into eukaryotic cells via receptors. Wagner *et al.* (12) have shown that the level of expression of genes targeted to the transferrin receptor in various cell lines in culture, using a transferrin–poly(L-lysine) conjugate as a carrier, correlates with the formation of DNA/transferrin–poly(L-lysine) complexes 80–100 nm in diameter. While this size DNA complex may be appropriate for uptake by the transferrin receptor of cells in culture, endocytic receptors usually discriminate against ligands of a determined size range *in vivo* (13–15). In addition, the size and/or structure of the DNA complex may be important in other steps involved in gene transfer—such as stability while in the circulation, receptor–ligand interaction, endocytosis, membrane permeabilization, endonuclease resistance, and nuclear targeting of the DNA complexes—that are required for the expression of a newly introduced gene in quiescent cells *in vivo*.

We report here a gene targeting system in which a ligand for the asialoglycoprotein receptor in liver hepatocytes [galactosylated poly(L-lysine)] is complexed to a plasmid DNA in the presence of increasing concentrations of NaCl. The condensation process is monitored by circular dichroism (CD) spectroscopy and electron microscopy (EM) and results in DNA/ligand–poly(L-lysine) complexes of small, defined size (10–12 nm). Using this procedure we were able to reproducibly target genes to the livers of adult rats and demonstrate expression of the genes in the liver for as long as 140 days (the duration of the experiment). The basic principle in preparing the DNA complex described in this paper involves the condensation of individual DNA molecules into *unimolecular* complexes of DNA with defined structure.

MATERIALS AND METHODS

Reporter Genes and Plasmid Preparation. The PEPCK–hFIX plasmid containing the human factor IX (hFIX) cDNA under the transcriptional control of the promoter regulatory region (nucleotides −460 to +73) of the phospho*enol*pyruvate carboxykinase (PEPCK) gene has been described (5). The plasmids were grown in *Escherichia coli* DH5α, extracted, and purified by standard techniques (16). No con-

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Abbreviations: FIX, factor IX; hFIX, human FIX; PEPCK, phospho*enol*pyruvate carboxykinase.

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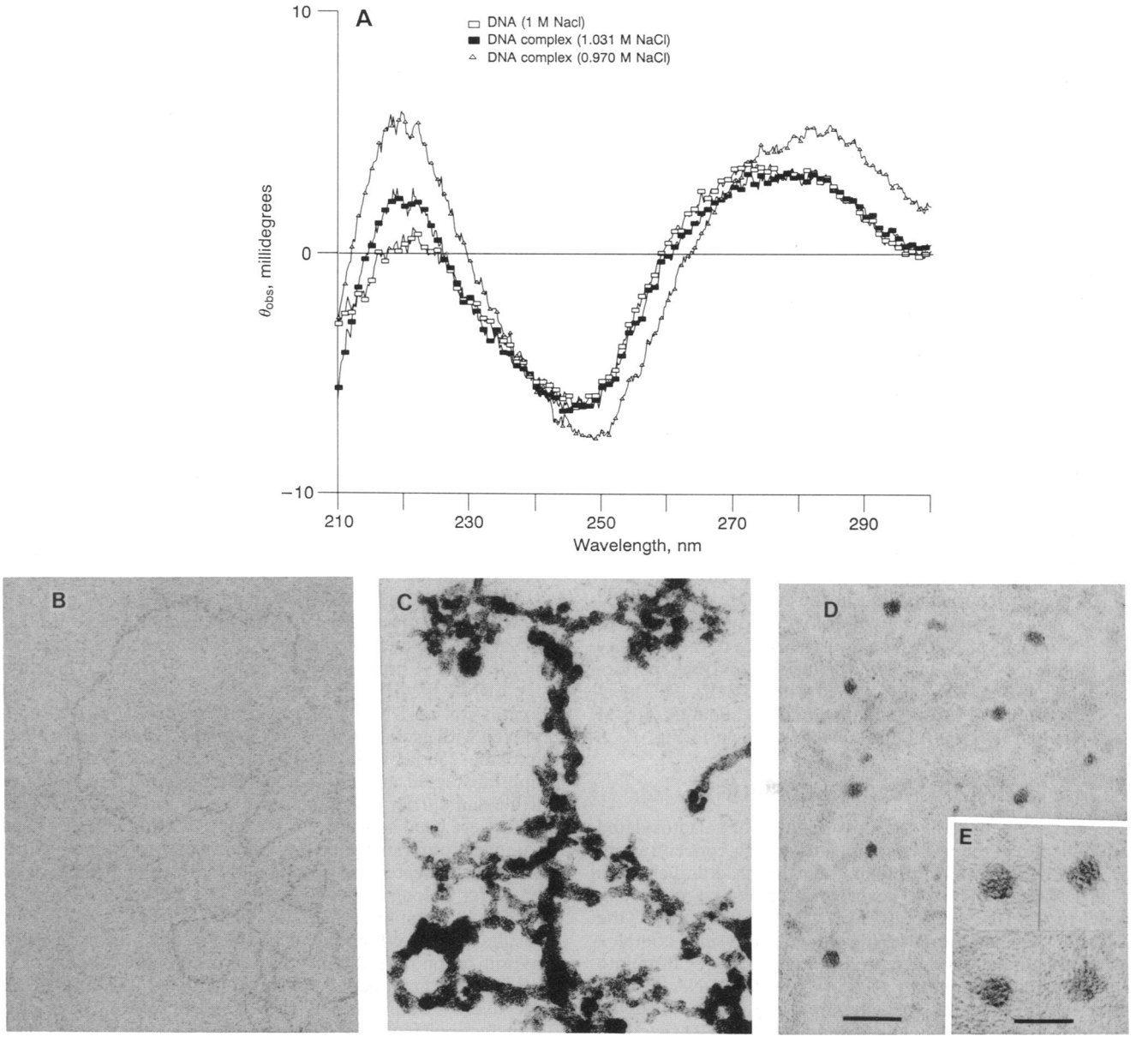

FIG. 1. Physical characterization of the galactose–poly(L-lysine)/DNA complexes. (*A*) CD spectra of the interaction of poly(L-lysine) and PEPCK-hFIX at increasing concentrations of NaCl. □, DNA in 1 M NaCl; ■, DNA complex as in *D* and *E*; △, DNA complex as in *C*. The spectra were obtained on a Jasco (Easton, MD) 600 spectropolarimeter with a 0.1-cm cuvette. The spectrum of the buffer was subtracted from each sample. (*B*) Electron micrograph of uranyl acetate-stained uncomplexed DNA (1 μg/ml). The grid was subjected to glow discharge prior to staining. A drop of DNA solution was added to the grid, blotted, and stained with 1% uranyl acetate (19). (*C*) Uranyl acetate staining of the DNA complex (10 μg/ml) prepared as above at a suboptimal concentration of NaCl (0.97 M). (*D*) Uranyl acetate staining of DNA complex (10 μg/ml) prepared as above at an optimal concentration of NaCl (1.031 M). (*E*) Same as in *D*; four complexes of DNA were selected and printed at higher magnification. Samples were diluted in water prior to preparation for EM. [Bar in *D* (applies *A–D*) = 33.3 nm; bar in *E* = 16.6 nm.]

taminating bacterial genomic DNA or RNA was present in the plasmid preparations.

Rats. Adult male Sprague–Dawley rats weighing approximately 300 g were anesthetized with ether. With aseptic technique, 0.3–0.5 ml of a solution containing 300 μg of DNA complex was injected into the caudal vena cava. The rats were killed at various times after infusion of the complex; blood samples and various organs were taken for analysis. The animal research protocol was reviewed and approved by the Case Western Reserve University Institutional Animal Care Committee.

Measurement of hFIX. Blood samples were assayed for hFIX procoagulant activity. A modification of the one-stage, partial thromboplastin time assay with FIX-deficient human plasma was used (17). The activity of hFIX in the plasma of rats injected with the DNA complex was compared with the functional activity of pooled plasma from 24 normal men.

Detection of mRNA and DNA by PCR. Total hepatic RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase and the resultant cDNA amplified by PCR. One microgram of the RNA was treated with 10 units of RNase-free DNase I, added to a solution containing 500 nM of $(dT)_{16}$ primer and 500 nM each dNTP, and heated at 42°C. One microliter of the cDNA pool was amplified by PCR using primers from the 5′ untranslated region of the PEPCK promoter (5′-TTGGCCAACAGGGGAAAG-3′) and the hFIX cDNA (5′-ATGTTCTGTCTCCTCAATATT-3′). As a control, the same RNA samples not converted to cDNA by reverse transcriptase were also amplified by PCR to ensure that contaminating plasmid DNA had not been amplified. The

products were separated by agarose gel electrophoresis and Southern blot hybridization was performed with a radiolabeled 700-bp *Ava* I–*Hin*dIII fragment of hFIX cDNA as a probe.

Production of the Galactosylated Poly(L-lysine) Carrier. Poly(L-lysine) was galactosylated essentially as described (18). In brief, 2 mg of poly(L-lysine) hydrobromide (average chain length, 100; Sigma P 7890) was reacted with 85 μg of α-D-galactopyranosyl phenylisothiocyanate (Sigma G-3266). The solution was adjusted to pH 9 by the addition of 0.1 volume of 1 M sodium carbonate (pH 9). The tube was shielded from light by aluminum foil and the solution was mixed for 16 hr at room temperature and then dialyzed in Spectra-Por (Spectrum Medical Industries, Houston) dialysis tubing (molecular weight cutoff, 3500) against 500 ml of 5 mM NaCl for 2 days with four changes per day. The reaction resulted in the galactosylation of 0.8–1% of the amino groups present in the solution, as determined by A_{250}.

Production of DNA Complex. Three hundred micrograms of PEPCK-hFIX contained in 150 μl of 700 mM NaCl was vortexed at medium speed in a Vibrax (Ika-Werk Instruments, Cincinnati) apparatus. Ninety-five micrograms of α-galactopyranosyl phenylisothiocyanate/poly(L-lysine) biconjugate dissolved in 150 μl of 700 mM NaCl was added dropwise to the vortexing solution of DNA. The slow addition of the polycation resulted in the formation of a turbid solution that was dissolved by the slow, stepwise addition of 3-μl aliquots of 5 M NaCl. The disappearance of the turbidity was monitored by eye and the solutions of DNA/poly(L-lysine) complexes were investigated with a JEOL-100C electron microscope. Further addition of 2-μl aliquots of 5 M NaCl resulted in the structural changes shown in Fig. 1.

RESULTS AND DISCUSSION

DNA was condensed by the slow addition of galactosylated poly(L-lysine) in the presence of NaCl at high concentration. CD spectroscopy was used to monitor the structural changes in the DNA complex that occurred after binding of poly(L-lysine) to the DNA moiety (Fig. 1*A*). At 0.97 M NaCl, the optical activity of the DNA/ligand–poly(L-lysine) complex indicated light scattering, representative of aggregation into multimolecular complexes (7–9). When the DNA/ligand–poly(L-lysine) complex was prepared at 1.03 M NaCl, the light-scattering component disappeared and the CD spectrum was indistinguishable from that of double-stranded DNA in 1 M NaCl (10, 11). The structure of the complex associated with each CD spectrum was determined by EM. When the ionic strength of the DNA/ligand–poly(L-lysine) complex was increased, the complex proceeded from an aggregated (Fig. 1*C*) to a condensed state (Fig. 1 *D* and *E*). When the ionic strength was increased above the critical range required for the condensation, the complex assumed a nonfunctional, rod-like conformation of increased size (J.C.P., T.F., and R.W.H., unpublished data). The diameter of the DNA complex observed in Fig. 1*D* (about 10 nm) conforms with the discrimination range necessary for internalization of molecular ligands by the asialoglycoprotein receptor (13–15).

The degree of condensation of the DNA complex is dependent on a number of variables, including the chain length of the poly(L-lysine), as well as the size, sequence, and state of the DNA—i.e., supercoiled, nicked, or linear (20–22). We have noted a critical concentration of NaCl required for condensation of a particular plasmid with poly(L-lysine) of a specific length; the concentration of NaCl used to affect the appropriate degree of condensation is dependent on the variables listed above. However, we have found that the formation of the particles shown in Fig. 1 *D* and *E* correlates with the prolonged expression of the transgene in the livers of intact rats. A more detailed discussion of the physical properties involved in the generation of a functionally active DNA complex will be presented in a subsequent report (J.C.P., T.F., and R.W.H., unpublished data).

We tested the ability of the DNA complexes to transfer DNA into the liver of adult rats. A chimeric gene composed of the PEPCK promoter (nucleotides −460 to +73) linked to the cDNA for hFIX (5) was used to follow the uptake and expression of DNA in the liver. DNA (300 μg) was complexed with galactosylated poly(L-lysine) (Fig. 1) and infused into the caudal vena cava of 12 intact rats. Plasma from nontransfected and mock-transfected animals, in which an irrelevant carrier was complexed to the PEPCK–hFIX plasmid, were used as controls. Western blot analysis of plasma samples from transfected rats using an anti-hFIX monoclonal antibody indicated the presence of hybridizing protein in the blood for as long as 136 days (Fig. 2*A*). Ferkol *et al.* (5) have shown that expression of the PEPCK–hFIX gene *in vivo* results in the production of a doublet as detected by Western hybridization, possibly because of differential processing of hFIX in rat hepatocytes. We also detected hFIX mRNA in the livers of animals analyzed 4, 6, 8, and 12 days after transfection (Fig. 2*B*). The same PEPCK–hFIX gene, when introduced into the livers of intact rats by an alternative method of receptor-mediated gene transfer that targeted the

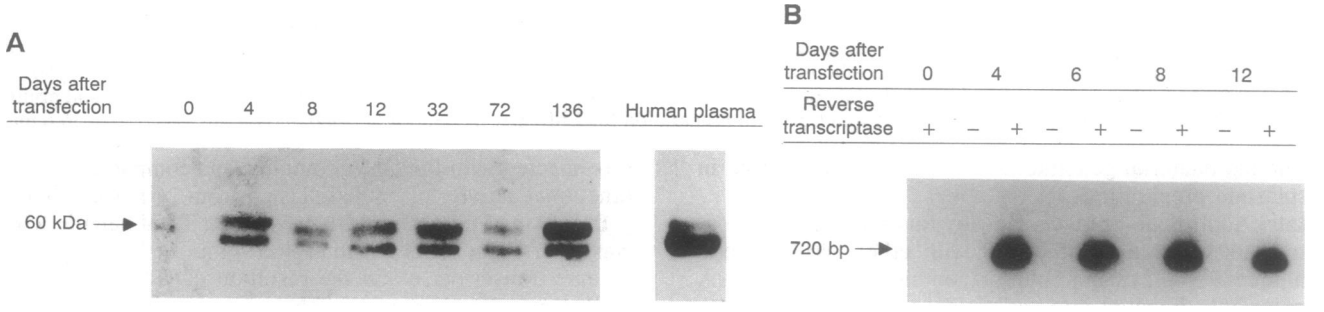

FIG. 2. Time-course of gene expression *in vivo*. Twelve adult rats injected in the caudal vena cava with 0.3–0.4 ml of a solution containing 300 μg of the PEPCK–hFIX gene complexed with galactosylated poly(L-lysine) were killed at the times indicated and tissues and blood samples were taken. Data are representative of 12 different animals injected in three experimental sets using two preparations of galactose–poly(L-lysine) biconjugate and four preparations of PEPCK–hFIX DNA. (*A*) Western blot hybridization of plasma samples (1 μl) from individual animals at various times after transfection with the carrier/DNA complex. Plasma from a mock-transfected animal (lane 0) and human plasma (1:4 dilution) from a normal individual were analyzed in parallel. Western hybridization was performed as described (5). (*B*) Presence of hFIX mRNA in livers of rats transfected with the PEPCK–hFIX gene was determined by Southern blot hybridization of the DNA generated by PCR amplification of the reverse-transcribed RNA. Hepatic mRNA from transfected rats was incubated in the presence (+) or absence (−) of reverse transcriptase. The resultant cDNA was amplified by PCR using primers specific for hFIX and PEPCK. The reaction products were separated by electrophoresis in a 1.6% agarose gel and analyzed by Southern blot hybridization using a radiolabeled hFIX cDNA probe.

asialoglycoprotein receptor, was detected for only 2 days after transfection (5). This suggests that the prolonged expression of the transfected DNA noted in the present study was not due to specific DNA sequences that stabilize the episomes in the nucleus or that allow replication of the transgene in the hepatocytes.

The activity of hFIX in the plasma of transfected rats was analyzed by a partial thromboplastin time assay on plasma obtained at various times after administration of the PEPCK–hFIX gene (17). Again, nontransfected animals were used as controls in each experiment. Biologically active hFIX at 15 ng/ml (72 days after transfection) to 1050 ng/ml (48 days after transfection) was produced in individual rats injected with the DNA complex (Table 1). These values range from 0.1% to 35% of the activity of FIX in normal human plasma. If the higher levels of hFIX noted in this study were produced in a patient with severe Christmas disease, we would predict a significant improvement in the coagulation status of the affected individual. The sources of variability in the level of expression of hFIX in the animals noted in this study are unclear; the variation could be due to either variability in the stability of the DNA complex *in vivo* or differences in transfer efficiency or expression in the target cells. Differences in receptor availability on the surface of hepatocytes or variations in the biological assay from animal to animal may partly account for the wide range of concentrations of hFIX expressed by the rats. We have performed statistical analysis on rats that were transfected with a plasmid containing the *Photinus pyralis* luciferase gene under the control of the simian virus 40 early promoter and enhancer elements and then were killed 6 days after injection of the DNA complex. The livers of these three animals had 11,600 ± 2340 integrated light units of luciferase activity per milligram of protein extract. This suggests that the variability in hFIX activity observed in Table 1 is not due to differences in the efficiency of gene transfer.

Transcription from the PEPCK promoter in the livers of transgenic mice can be induced by the administration of a high-protein, carbohydrate-free diet (23, 24). To investigate the regulation of the newly introduced gene, we analyzed the blood of transfected animals for the presence of hFIX by Western blot hybridization before and after feeding a high-protein, low carbohydrate diet or a standard chow diet for 1 week (Fig. 3*A*). Expression of the PEPCK–hFIX gene was increased from 2.8- to 3.6-fold by the high-protein diet 8, 12, 80, and 140 days after injection of the DNA complex.

To investigate whether the DNA had undergone random integration into the genome of transfected animals, hepatic DNA isolated from a rat 32 days after transfection and from a control rat was digested with *Bgl* II, which produces an intact, linear form of the plasmid (4.5 kb), and with *Eco*RI,

Table 1. hFIX activity in plasma from rats transfected with the PEPCK–hFIX gene

Exp.	Rat	Sampling time, days	hFIX activity, unit/ml
A	1	2	0.040
	2	2	0.045
	3	4	0.045
	4	4	0.025
B	5	6	0.330
	6	8	0.135
	7	12	0.160
	8	12	0.075
	9	32	0.125
C	10	48	0.350
	11	72	0.005
	12	136	0.105

The relative concentration of hFIX in the blood of rats treated with the DNA complex was evaluated. One unit of FIX activity in 1 ml of normal human plasma is equivalent to 100% functional activity or ≈3 μg of hFIX per ml. Background FIX activity in the rat plasma was determined in each assay by using plasma from untransfected controls and was subtracted from the FIX activity of transfected rats. The average background of FIX activity for the control animals was 79.5%, 42.6%, and 56.8% of the experimental value in Exp. A–C, respectively.

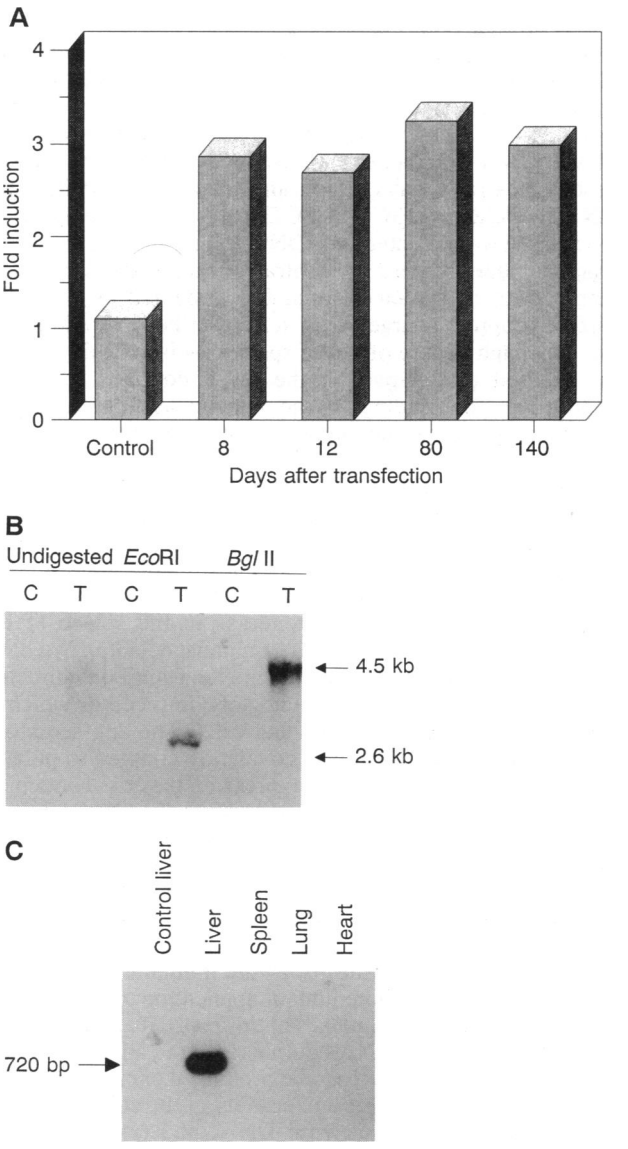

FIG. 3. Transcriptional regulation and specificity of the gene transfer system. (*A*) Transfected rats were fed a high-protein, carbohydrate-free diet for 1 week. Blood samples were taken at the initiation of the treatment and after 1 week on the diet and analyzed by Western blot hybridization as indicated in the legend for Fig. 2. The animals at the 8- and 12-day points were compared with transfected rats that were fed a standard chow diet. Data were obtained by densitometric analysis of Western blot photographic films and indicate fold increase in hFIX after dietary treatment compared with pretreatment (control). (*B*) Detection of exogenous plasmid DNA by Southern blot hybridization. The liver from an animal 32 days after transfection was taken and genomic DNA was isolated. Five micrograms of total DNA from transfected rats and from a nontransfected control was digested with either *Eco*RI or *Bgl* II and the fragments were separated by electrophoresis in a 1.6% agarose gel before Southern blot analysis. (*C*) PCR analysis was performed with total genomic DNA isolated from spleen, lung, heart, and liver taken from a rat 6 days after transfection with 300 μg of the DNA complex.

which liberates the 2.6-kb chimeric gene from the plasmid. *Eco*RI digestion produced a single band of 2.6 kb, the size of the PEPCK–hFIX gene; *Bgl* II digestion generated a single band consistent with the size of the linear plasmid (Fig. 3*B*). This indicates that the DNA was present in the liver as an episome. However, we cannot rule out the possibility that a fraction of the transfected DNA was randomly integrated into the hepatic genome and could not be detected at the level of resolution of the Southern analysis.

The asialoglycoprotein receptor is present only in parenchymal cells of the liver. However, asialoglycoproteins and other galactose-terminal glycoproteins can also be taken up by macrophages, depending on the size of the molecular ligand (13–15). To investigate the tissue targeting specificity of the DNA complexes, we isolated DNA from various tissues of a rat 6 days after transfection and amplified the DNA by PCR (Fig. 3*C*). hFIX DNA was found only in the liver; there was no detectable DNA in lung and spleen, which contain macrophages. In contrast, transfected DNA was detected in the spleen of animals transfected by using the asialoglycoprotein targeting system described by Ferkol *et al.* (5). This high degree of tissue specificity is probably due to the method of compacting the DNA complex to a size appropriate for binding to the hepatic asialoglycoprotein receptor, and it may be important for the targeting of genes to other receptors.

We have tested the ability of this method to direct DNA to other receptors by substituting mannose for galactose in order to target the mannose receptor present in macrophages. Animals injected with DNA complexes containing the *P. pyralis* luciferase gene and mannosylated poly(L-lysine) produced significant levels of luciferase in the spleen (T.F., J.C.P., F. Mularo, and R.W.H., unpublished data). We have also used an Fab fragment of a monoclonal antibody directed against the rat polymeric immunoglobulin receptor which is expressed in the airway epithelial cells. The anti-secretory component Fab antibody was covalently coupled to poly(L-lysine) and complexed to the expression plasmid containing the luciferase gene by the procedure described in this paper. Rats injected with the DNA complex had transgene expression for as long as 12 days (the duration of the experiment), but only in tissues that expressed the receptor (T.F., J.C.P., C. Kaetzel, R.W.H., and P. Davis, unpublished data). These findings underscore the flexibility of this system for delivering DNA to specific tissues of an adult animal.

This procedure has potential for application to human gene therapy. The major advantages of this method are (*i*) the ease of preparation of the DNA complex; (*ii*) the ability to target genes to specific tissues; (*iii*) the prolonged expression of the gene in the liver; (*iv*) the relative safety of the complex, since it is devoid of infectious viral DNA; and (*v*) the relative stability of the DNA as an episome in the nucleus. We have recently shown that two injections of a plasmid containing a chimeric PEPCK–human low density lipoprotein receptor gene, complexed to the carrier described in this paper, into the ear vein of a hypercholesterolemic Watanabe rabbit, resulted in a 38% decrease in the level of total cholesterol in the blood of the rabbit 28 days after the administration of the DNA complex (M. Molas, J.C.P., T.F., and R.W.H., unpublished results). While still preliminary, these findings, together with the results shown in the present paper, indicate the potential of this technique for correcting genetic defects.

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